EXHIBIT RAS-8

This is exhibit RAS-8 referred to in Declaration Under 37 C.F.R. 1.132 by Richard Anthony Strugnell dated 27.9.01

Richard Strugnell

forward, is easy to perform, and incorporates 100 μg or less of immunogen. With this or any other immunization technique, several factors need be considered. These include selection of animal species, time to harvest antibody of highest sensitivity and specificity, selecting the appropriate time to reimmunize the animals, as well as incorporating an appropriate immunologic technique for screening the antisera for titer, specificity, and sensitivity.

[3] Production of Specific Antisera by Immunization with Precipitin Lines

By JENS KRØLL

For the production of monospecific antisera it is essential that the antigens used be as pure and native as possible. One simple way to meet these requirements is to use specific antigen–antibody complexes as immunogen.[1] Passive immunodiffusion techniques can be used for this purpose.[1–7] However, these techniques are insufficient for the resolution of complex antigen–antibody systems. This requirement is better met by the more recently developed quantitative immunoelectrophoretic procedures, which in addition to a higher resolution improve the conditions for the comparison of different patterns.[8–18]

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The following sections deal with the use of the line-immunoelectrophoretic procedure for the isolation of pure immunogens as well as for the evaluation of antibody titers and specificity.[14–18]

Materials and Methods

Line Immunoelectrophoresis. This procedure is carried out as described elsewhere in this volume [25].

Isolation of Precipitin Lines. After immunoelectrophoresis the agarose gel is blotted with filter paper under a slight pressure to remove nonprecipitated antigens and to reduce the agarose gel to a thin but not completely dry sheet. The precipitin lines visualized by dark-field illumination or by staining in a dilute aqueous solution of Coomassie Brilliant Blue (0.1 g/liter) are cut out from the gel by means of a Linocutter (Fig. 1). The 8–10 cm-long narrow gel strip containing the precipitin line is transferred to a 5-ml test tube and washed three times with isotonic saline to elute remaining nonprecipitated or weakly associated antigens from the precipitate. Between washes the gel is centrifuged at 15,000 *g* for 10 min.

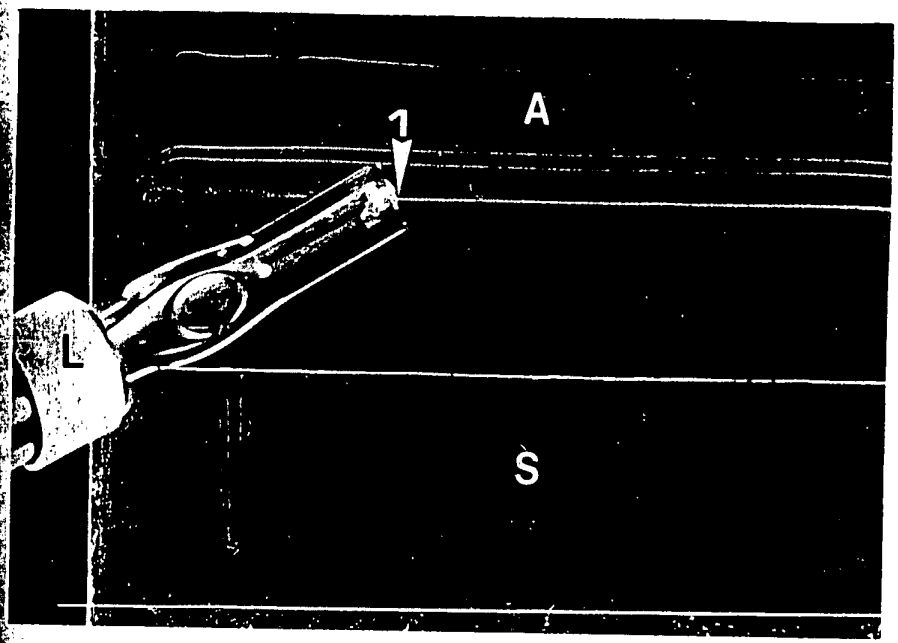

FIG. 1. Isolation of precipitin lines. S, Sample gel (1 × 20 × 70 mm) containing 0.4% of human serum; A, antiserum gel containing 3% of a polyspecific antiserum against human serum proteins. Immunoelectrophoresis was carried out at 1.5 V/cm for 20 hr. Anode is at top. The precipitin lines are visualized by dark-field illumination. One of the lines (1) is partially cut out from the gel by means of the Linocutter (L).

Immunization. The whole of the washed agarose gel strip containing the immunocomplex is solubilized by suspension in 1 ml of 6 *M* KI. After complete solubilization 0.25 ml of this solution is vortex-mixed with an equal volume of Freund's adjuvant (complete adjuvant for the first inoculation, incomplete for the following ones). Of this mixture, 0.5 ml, which contains approximately 2 cm of dissociated precipitin line (corresponding to 0.1–5 μg of antigen, depending on the density of the line), is used for each of four inoculations given subcutaneously to rabbits every second week. Blood is collected 8–10 days after the last inoculation. After the attainment of a specific immune response the rabbits should be reinoculated only at signs of a decrease in antibody titer, preferably with precipitates developed against the monospecific antiserum.

Estimation of Antiserum Titers and Specificity.[15,17,18] The line immunoelectrophoretic technique is used for the determination of antiserum titers as illustrated in Fig. 2. Small samples of antiserum are placed in front of the antigen-containing gel section. The deflection of the precipitin line caused by local absorption of antigen at the well during immunoelectrophoresis is proportional to the titer of the antiserum.[17] The specificity of the antiserum can be tested by absorption against the corresponding polyspecific antiserum (Fig. 3). Alternatively, the titer and specificity of different antisera can be compared by correlation of line patterns developed side by side as shown in Fig. 3. Here the antiserum titer is inversely proportional to the distance of antigen migration from the origin to the level of precipitation.

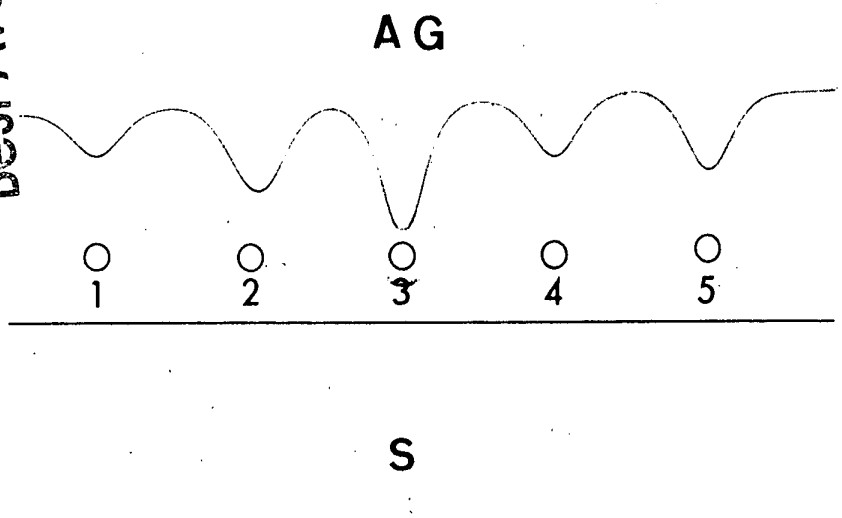

FIG. 2. Estimation of antiserum titer. S, Sample gel (1 × 20 × 80 mm) containing 0.1% of human serum; AG, antiserum gel containing 2% of antitransferrin serum raised by immunization with transferrin precipitin line. 1–5, Circular wells (diameter 2 mm); wells 1–3 contain 1, 2, and 3 μl of monospecific antitransferrin reference antiserum diluted 1 : 10; wells 4 and 5 contain 2-μl samples of different undiluted sera from rabbits immunized with transferrin precipitin lines. Immunoelectrophoresis was carried out at 1.5 V/cm for 20 hr. Anode is at top. It appears that the cathodic deflection of the precipitin line above the reference antiserum wells (1–3) is directly proportional to the amount of antiserum added to the well. Accounting for the difference in dilution, the deflections caused by the precipitin line antisera (4 and 5) show that the titer of these antisera are about 1 : 15 (4) and 1 : 10 (5) of the titer of the reference antiserum.

Comments

Excision of precipitin lines from crowded patterns as developed from human serum against the corresponding polyspecific antiserum will often result in the inclusion of unwanted antigens in the isolated gel strip due to partial fusion of different lines or trailing phenomena caused by overloading with a dominant antigen (e.g., serum albumin).

The separation of individual lines can be improved by immunoelectrophoresis against an antiserum gradient or by use of an oligospecific antiserum (e.g., the antiserum obtained by immunization with a not sufficiently purified immune complex).

Trailing phenomena can be reduced by avoidance of overloading and by the use of a low concentration of agarose in the immunoelectrophoresis gel.[15] The occasional nonspecific binding of some antigens to the gel matrix or to other antigens can usually be avoided by incorporation of a nonionic detergent (e.g., 1% Triton X-100) in the agarose gel. Use of the Linocutter for isolation of the precipitin lines enables the isolation of very narrow gel strips and thus improves the conditions for the isolation of a pure immunogen.

Occasionally crossed immunoelectrophoresis in the first-dimensional separation of the antigens can improve the conditions for the isolation of well separated precipitin peaks. However, in crowded patterns overcrossing of different precipitin lines reduces the yield of pure immunogen.

In the production of specific antisera against serum proteins from other species, cross-reactions with specific antisera against human serum proteins can be used for identification.[14]

The antigen content in the weakest precipitin lines detectable by dark-field illumination is approximately 50 ng/cm. This, or even smaller amounts of antigen, is sufficient to induce a specific immune response in rabbits illustrating the high antigenicity of immunocomplexed antigens.[8,14] Solubilization of the gel strip containing the immunocomplex in saturated

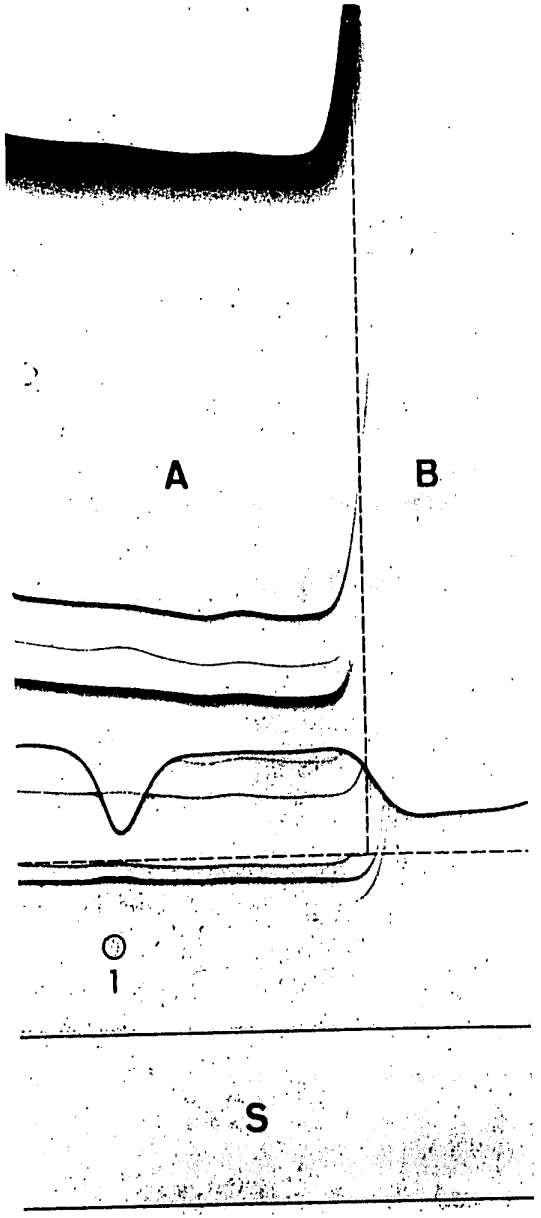

FIG. 3. Test of antiserum specificity. S, Sample gel (1 × 20 × 60 mm) containing 0.1% of human serum; A and B, antiserum gel sections (dashed border line). Section A contains 1.5% of a polyspecific antiserum against human serum proteins; section B contains 2% of antitransferrin serum raised by immunization with transferrin precipitin line. 1, Application well (diameter 2 mm) containing 2 μl of the antitransferrin serum used in section B. Immunoelectrophoresis was carried out at 1.5 V/cm for 20 hr. Anode is at top. Monospecificity of the antiserum tested is evidenced from the appearance of one line only in antiserum gel section B. Also this line is the only one in section A, showing a cathodic deflection due to local absorption at the well (1).

KI has not proved to be essential for the immune response but facilitates handling of the immunogen.

Acknowledgment

The skillful technical assistance of Anna Margrethe Poulsen, Birthe Larsen, Lene Ahrenst, and John Post is gratefully acknowledged.

[4] Polymers for the Sustained Release of Macromolecules: Their Use in a Single-Step Method of Immunization

By ROBERT LANGER

Since their introduction by Freund in 1951,[1] water-in-oil suspensions containing antigen have become standard immunological adjuvants. However, poor degradation of the mineral oil base restricts their use to experimental animals. Therefore, safer adjuvants with comparable effectiveness have been sought for use in human immunization.[2] Although these antigen-dispersing vehicles enhance antibody production when compared to injection of free material, they fall short of levels attained with the help of Freund's complete or incomplete (minus bacteria) adjuvants. In 1976, we developed polymeric delivery systems that permitted the sustained release of molecules as large as 2,000,000 daltons for up to 4 months.[3,4] More recently, it was demonstrated that single polymer implants in mice could release antigen continuously and stimulate antibody formation for more than 25 weeks.[5] These studies suggest that polymeric delivery systems could provide a simple, safe, and effective single-step immunization.

This report concerns the methodology for fabricating and utilizing these polymeric systems; it includes four main sections: methods of preparing polymeric delivery systems; methods of regulating the release kinetics of these systems; results of immunization tests with these delivery systems; and discussion of the advantages and limitations of the systems as well as potential directions for future research in this area.

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